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Effect of soybean meal replacement by cottonseed meal and iron supplementation on growth, immune response and resistance of Channel Catfish (*Ictalurus puctatus*) to *Edwardsiella ictaluri* challenge

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Abstract

Three basal diets containing 0%, 27.5% and 55.0% solvent-extracted cottonseed meal (CSM) as replacements of 0%, 50% and 100% of solvent-extracted soybean meal (SBM) on an equal nitrogen basis were each supplemented with three levels of iron (40, 336 and 671 mg/kg) from ferrous sulfate heptahydrate (3 × 3 factorial experiment). Each diet was fed to juvenile channel catfish in triplicate aquaria twice daily to apparent satiation for 10 weeks for subsequent determination of growth response, hematology, specific and non-specific immune response, and mortality following *Edwardsiella ictaluri* challenge. Fish fed diets containing 27.5% CSM as a replacement of 50% of SBM had improved weight gain (WG) and feed efficiency ratio (FER). Total replacement of SBM by 55.0% CSM decreased WG, feed intake (FI) and FER. Total cell count (TCC), red blood cell count (RBC), hematocrit (Ht) and hemoglobin (Hb) were not affected by dietary levels of CSM. Iron supplementation significantly affected TCC and RBC and maximum values of these parameters were obtained at 336 mg of iron/kg diet. However, Ht and Hb were not affected by increasing levels of supplemental iron. Values for TCC, RBC and Hb were significantly affected by the interaction between dietary levels of CSM and iron. For fish fed the diet containing 0% CSM (SBM-based diets), these parameters increased linearly with increasing dietary levels of iron. When CSM levels were increased to 27.5% or higher, 336 mg supplemental iron was sufficient for maximum hematological values. Macrophage chemotaxis in the presence of exoantigen was significantly higher for fish fed diets containing 55.0% CSM as compared to those fed the lower CSM diets. Agglutinating antibody titers were also significantly higher for fish fed diets containing CSM, but the values did not differ for

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those fed the 27.5% or 55.0% CSM diets. Dietary levels of iron, and interactions between dietary levels of iron and CSM had no effect on macrophage chemotaxis and antibody titers. Cumulative mortality at 15 days post-challenge was significantly higher for fish fed the SBM-based diet (0% CSM) at 54.4% as compared to 35.0% and 21.6% for those fed the 27.5% and 55.0% CSM diets, respectively. No differences were observed among mortality of fish fed the CSM-containing diets. Dietary levels of iron supplementation, and the interactions between dietary levels of iron and CSM had no effect on post-challenged mortality of fish.

Keywords: Iron; Cottonseed meal; Soybean meal; Growth response; Chemotaxis; Antibody titers; Disease resistance

1. Introduction

Nutrition has long been recognized as a key factor in host defense against pathogens. Research on the influence of nutritional factors on the resistance of terrestrial animals to infectious diseases has progressed rapidly in recent years. However, interrelationships between nutrition, immunity and disease resistance in fish are poorly understood. For channel catfish, available information on this subject has been limited to ascorbic acid (Li and Lovell, 1985; Li et al., 1993), fatty acids (Blazer et al., 1989; Fracalossi and Lovell, 1994), vitamin E (Wise et al., 1993), folic acid (Duncan and Lovell, 1994), zinc (Paripatananont and Lovell, 1995; Lim et al., 1996a), iron (Sealey et al., 1997; Lim and Klesius, 1997) and selenium (Wang et al., 1997).

Soybean meal is currently the most commonly used plant protein source in fish feeds and comprises up to 50% of the diet of channel catfish (NRC, 1993). Replacement of soybean meal with less expensive plant protein sources would be beneficial in reducing feed costs. Cottonseed meal, which ranks second to soybean meal in the United States in terms of tonnage produced, is less expensive than soybean meal on a per unit protein basis (Robinson and Li, 1995). Numerous studies have been conducted to determine the level of cottonseed meal that can be incorporated in channel catfish diets without affecting their growth performance (Dorsa et al., 1982; Robinson and Rawles, 1983; Robinson et al., 1984; Robinson and Daniels, 1987; Robinson and Li, 1994; Robinson and Tiersch, 1995). Results have shown that the amount of cottonseed meal that can be included in catfish diets depends mainly on the levels of free gossypol and available lysine. Robinson (1991) reported that prepressed solvent-extracted cottonseed meal can replace up to 50% of soybean meal in juvenile channel catfish diets without requiring lysine supplementation and up to 100% of soybean meal if supplemental lysine is used.

Free gossypol, when present in large quantity in the diet, has been shown to be toxic to monogastric animals including fish. Growth depression occurred in channel catfish fed diets containing more than 900 mg free gossypol/kg diet (Dorsa et al., 1982), whereas a diet containing as low as 290 mg free gossypol/kg diet reduced the growth of rainbow trout (Herman, 1970). Iron, as ferrous sulfate, has been successfully used to counteract the toxicity of free gossypol in diets of monogastric, terrestrial animals (Jones, 1987). High levels of supplemental iron used to counteract the toxicity of gossypol may be harmful to fish because it has been suggested that a delicate balance exists between the need of iron for host defense mechanisms and the need of iron to sustain microbial growth. Sealey et al.

(1997) reported that high levels of dietary iron may lead to increased susceptibility of channel catfish to *Edwardsiella ictaluri* infection. Moreover, no studies have been conducted to evaluate the efficacy of this compound to detoxify gossypol in fish feeds containing cottonseed products. Therefore, this study was undertaken to evaluate the effect of iron supplementation to diets containing different levels of cottonseed meal on growth, hematology, immune response and resistance of juvenile channel catfish to *E. ictaluri* challenge.

2. Material and methods

2.1. Experimental diets

Nine experimental diets were formulated to contain approximately 32% crude protein and 2900 kcal of digestible energy/kg diet based on feedstuff values reported by NRC (1993). The diets consisted of three basal diets (1, 2 and 3) containing 0%, 27.5% and 55.0% of cottonseed meal (CSM) (0.122% free gossypol), respectively, as replacements of 0%, 50% and 100% of soybean meal (SBM) on a nitrogen basis (Table 1). Since SBM and

Table 1
Percentage composition of basal experimental diets containing different levels of cottonseed meal

Ingredients	Percent in diet		
	1	2	3
Menhaden fish meal	8.0	8.0	8.0
Soybean meal (48%)	45.0	22.5	–
Cottonseed meal (41%)	–	27.5	55.0
Corn meal	10.0	10.0	10.0
Wheat middlings	26.5	20.0	13.5
Cod liver oil	3.0	3.0	3.0
Dicalcium phosphate	1.0	1.0	1.0
Trace mineral mix[a]	0.5	0.5	0.5
Vitamin mix[b]	0.5	0.5	0.5
Carboxymethyl cellulose	3.0	3.0	3.0
Celufil	2.5	3.7	4.9
Lysine HCl	–	0.3	0.6
Percentage protein of soybean meal replaced by cottonseed meal	0	50.0	100
Free gossypol[c]	–	0.0336	0.0671
Determined iron content (mg/kg diet)	213.6	282.9	203.9

[a] Trace mineral mix provided the following minerals (mg/kg diet): zinc (as $ZnSO_4 \cdot 7H_20$), 150; iron (as $FeSO_4 \cdot 7H_2O$), 40; manganese (as $MnSO_4 \cdot 7H_2O$), 25; copper (as $CuCl_2$), 3; iodine (as KI), 5; cobalt (as $CoCl_2 \cdot 6H_2O$), 0.05; selenium (as Na_2SeO_3), 0.09.

[b] Vitamin mix provided the following vitamins (mg/kg diet unless otherwise stated): vitamin A, 4000 IU; vitamin D_3, 2000 IU; vitamin K, 10; vitamin E, 50; thiamin, 10; riboflavin, 12; pyridoxine, 10; panthothenic acid, 32; nicotinic acid, 80; folic acid, 2; biotin, 0.2; vitamin B_{12}, 0.01; choline chloride, 400; L-ascorbyl acid-2-polyphosphate (15% vitamin C activity), 400; celufil, 3823.8.

[c] Calculated from the free gossypol content of cottonseed meal.

CSM contain different levels of crude protein, fat and fiber, the level of wheat middlings was adjusted to compensate for these differences. The total lysine content of the basal diets was maintained equal by the use of L-lysine hydrochloride (about 80% lysine). Iron as ferrous sulfate heptahydrate at levels of 0, 296 and 631 mg/kg diet was supplemented to each of the basal diets at the expense of celufil (3 × 3 factorial experiment). Since the trace mineral mix contained 40 mg iron as ferrous sulfate/kg diet, the total levels of supplemental iron were 40, 336 and 671 mg/kg diet. The two highest levels of iron (336 and 671 mg) were added to counteract the effects of gossypol in diets containing 27.5% and 50.0% CSM on a 1:1 weight ratio of iron to free gossypol as has been suggested for swine and poultry (Jones, 1987). Gossypol content of cottonseed meal was analyzed by the Texas Agricultural Research and Extension Center, The Texas A&M University System, San Angelo, TX.

All ingredients were ground to pass through a 1-mm mesh screen and processed into 3-mm diameter pellets. Diet preparation and storage were as described by Lim et al. (1996b) except that the pellets were dried at room temperature to a moisture content of less than 10%. Proximate composition of the experimental diets (Table 2) was determined in triplicate following the methods of the AOAC (1990). Iron content of the three basal diets (Table 1) in which the added trace mineral mix contained 40 mg iron/kg was determined using an inductive-coupled argon plasma (ICAP) spectrophotometer following the methods of Campbell and Plank (1992).

2.2. Fish and feeding

USDA-ARS strain 103 channel catfish (*Ictalurus punctatus*) fingerlings from a single spawn, which had been maintained at the USDA Aquatic Animal Health Research Laboratory on a commercial diet to an average weight of 6.15 ± 0.05 g, were randomly stocked into 27 110-l aquaria at a density of 40 fish/aquarium. Aquaria were supplied with flow-through (0.6–1.0 l/min) dechlorinated tap-water. Water was continuously aerated and photoperiod was maintained at 12:12 h light/dark schedule. Water temperature and

Table 2
Proximate composition of experimental diets

CSM (% of diet)	Fe (mg/kg diet)	Nutrient (% dry matter)			
		Moisture (%)	Crude protein	Crude fat	Ash
0	40	7.07	34.68	5.95	7.48
	336	5.43	33.48	5.64	7.82
	671	5.44	32.54	5.44	7.81
27.5	40	5.81	32.49	5.48	7.61
	336	4.15	32.34	5.76	7.67
	671	6.34	32.39	6.31	7.58
55	40	6.22	33.37	5.11	7.56
	336	6.84	32.45	5.35	7.67
	671	6.71	32.03	6.21	7.63

CSM = cottonseed meal. Fe values include iron supplied by trace mineral mix.

dissolved oxygen were measured in three randomly selected aquaria three times/week using a YSI Model 57 Oxygen Meter (Yellow Springs Instrument, Yellow Spring, OH[1]). Water temperature ranged from 24.4 to 27.5 °C with an average of 26.0 ± 0.1 °C, and dissolved oxygen varied from 5.6 to 8.0 mg/l with an average of 6.7 ± 0.1 mg/l. The water contained less than 0.5 mg iron/l.

Each of the nine experimental diets was randomly fed to fish in triplicate aquaria twice daily (between 0730–0830 and 1430–1530 h) to apparent satiation for 10 weeks. At each feeding, the diet was offered by hand four to five times until apparent satiation was reached. Feed intake was calculated daily by the difference in diet weight before and after feeding. All aquaria were cleaned once weekly by scrubbing and siphoning of accumulated wastes. On cleaning days, fish were fed only once in the afternoon. Fish in all aquaria were counted and weighed collectively at biweekly intervals. No feeding was done on weighing days.

2.3. Hematological assay

Blood samples were obtained from fish at the end of week 10. Four fish/tank were randomly chosen and anesthetized with tricane methanesulfonate (MS-222, Argent Chemical Redmond, WA) at 125 mg/l. Blood samples were collected from the caudal vein using heparinized 27-gauge needles and tuberculin syringes (20 units/ml) for determination of hematocrit (Ht), total cell count (TCC), red blood cell count (RBC) and hemoglobin (Hb).

Hematocrit was determined by the microhematocrit method described by Brown (1988). Total cell and red blood cell counts were determined by diluting whole blood and enumeration in a hemacytometer. Hemoglobin was determined by the total hemoglobin kit (Sigma Diagnostics, Sigma, St. Louis, MO) which is a standardized procedure of the cyanomethemoglobin method. Hemoglobin values were adjusted by a cyanomethemoglobin correction factor for channel catfish as described by Larsen (1964).

2.4. Collection of peritoneal exudate cells

Collection and isolation of peritoneal exudate cells followed the procedure of Klesius and Sealey (1996). At the end of week 10, four fish from each of the three replicate tanks were randomly removed and injected intraperitoneally (IP) with 0.25 ml of squalene (Sigma) and transferred into 57-l aquaria where they continued to receive their respective experimental diets. Five to seven days later, they were removed, anesthetized with MS-222 and injected (IP) with 15 ml sterile, cold phosphate-buffered saline (PBS) solution. Using a 20-gauge needle attached to a three-way valve, the PBS was removed along with the squalene-elicited exudate cells into a 50-ml centrifuge tube. The peritoneal fluids of four fish from the same tank were combined and centrifuged at

[1] Use of trade or manufacture's name does not imply endorsement.

300 × *g* for 10 min. The supernatant was discarded, and the cells suspended in calcium-, magnesium- and sodium-free Hank's Balance Salt Solution (HBSS) without phenol red (Gibco, Grand Island, NY) for chemotaxis assay. Cell counts and viability were established following enumeration with a hemacytometer in 5% trypan blue counting solution.

2.5. Chemotaxis assay

Chemotaxis was determined by a modification of the lower-surface method of Boyden (1962) as described by Klesius and Sealey (1996). Assays were performed in triplicate using blind well chemotaxis chambers (Corning Co Star, Cambridge, MA) and 8-μm pore diameter polycarbonate membrane filters (Nucleopore, Pleasontan, CA) pre-soaked for 5 min in RPMI-1640 (Gibco) containing 1% horse serum. In the bottom of each chamber, 50 ml of *E. ictaluri* exoantigen (2.80 mg protein/ml) (Klesius, 1993) was added together with 150 μl RPMI-1640 + 1% horse serum (Gibco). In the bottom of the control chamber only RPMI + 1% horse serum was added. Peritoneal macrophages were added to the upper compartment of the chamber (separated from the bottom chamber by filter) at a concentration of approximately 5×10^5 cells/chamber. The chambers were incubated on a horizontal platform shaker (100 rpm) for 100 min at 25 °C. Following incubation, filters were removed, inverted, placed on a slide, attached with clear fingernail polish and stained with Leukostat. The number of macrophages on the surface of the filter was counted in five fields of triplicate filters at 100 × .

2.6. Agglutinating antibody titer assay

Sixteen days after *E. ictaluri* challenge, four surviving fish were randomly removed from each of the replicate tanks and blood samples collected following anesthetizing with MS-222. Approximately 100 μl serum/sample was collected following centrifugation at 1000 × *g* for 5 min then stored frozen at −80 °C for subsequent determination of agglutinating antibody titers to *E. ictaluri* by modifying the method of Chen and Light (1994). *E. ictaluri* (AL-75-94) were grown in brain–heart infusion broth (BHI) for 24 h and killed in 1% formalin. The cells were centrifuged at 3000 × *g*. The resulting cell pellet was washed twice in 0.85% saline solution and suspended in saline solution to an optical density of 0.8 at 540 nm. Beginning with a 1:10 dilution (10 μl of serum and 90 μl of PBS), twofold serial dilutions were then made in 96 round-bottom microtiter plates by adding 50 μl of diluted serum onto the remaining wells plated with 50 μl of PBS. Thereafter, an equal volume (50 μl) of bacterial suspension was added to each well; thus the initial dilution of serum was 1:20. The plates were covered with plastic film and incubated at room temperature for 16–18 h. The agglutination end point was established as the last serum dilution where cell agglutination was visible after incubation. Antibody titers are reported as the reciprocal of this serum dilution and transformed to $\log_{10}$ values. Positive and negative agglutination sera were used as assay controls.

Prior to experimental challenge, four fish from each of the three replicate tanks were also bled for measurement of serum agglutinating antibody titers and all fish were negative.

2.7. E. ictaluri challenge

To determine the optimum bacterial cell concentration to use in experimental challenge, groups of 20 fish (average weight 21.8 g) which were held in separate aquaria and fed the SBM-based diet supplemented with 40 mg iron/kg for 8 weeks were placed in 18.4-l perforated plastic buckets and immersed for 30 min in static, aerated aquaria containing suspensions of 10^6, 2×10^6, 4×10^6 and 8×10^6 *E. ictaluri* cells/ml. Water flow and feeding were discontinued for the first 24 h after challenge and mortality recorded twice daily for 15 days. The LC_{50} (concentration lethal to 50% of exposed fish), calculated by the Karber method (Plumb and Bowser, 1983), was 5×10^6 cells/ml and was the concentration used for the experimental challenge.

At the conclusion of week 10 of the feeding period, 20 fish from each tank (average weight ranging from 27.5 to 43.9 g) were randomly removed and challenged by immersion (Klesius and Sealey, 1995). *E. ictaluri* (AL-75-94), from a virulent outbreak of enteric septicemia of catfish, was grown in brain heart infusion (BHI) broth for 24 h (Klesius, 1992). Fish were placed in perforated 18.4-l plastic buckets and immersed for 30 min in static, aerated aquaria containing 5×10^6 cells/ml of *E. ictaluri*. Water flow and feeding were discontinued for the first 24 h after challenge. Mortality was monitored and recorded twice daily before feeding for 15 days.

2.8. Statistical analysis

Data were analyzed by a two-way analysis of variance using the general linear model procedure (SAS Institute, 1993). Duncan's multiple-range test was used to determine significant differences due to cottonseed meal, iron and cottonseed meal and iron interactions. If a significant interaction was observed, the differences between simple effects were determined by contrast. Differences were considered significant at the 0.05 probability level.

3. Results

Mean final weight gain (WG), dry matter feed intake (FI), feed efficiency ratio (FER) and survival are presented in Table 3. Mean survival of fish in all treatments ranged from 97.5% to 100% and was not significantly ($P > 0.05$) affected by dietary levels of CSM, iron or the interaction between CSM and iron. The average WG pooled by dietary CSM levels showed that fish fed the 55.0% CSM diets had significantly ($P < 0.05$) lower WG than the groups fed diets without or with 27.5% CSM. Dietary levels of iron had no significant effect on WG but the interaction between CSM and iron was significant. Among diets containing no CSM, the response of fish to increasing levels of dietary iron was linear. For diets supplemented with 40 and 336 mg iron/kg, the response of fish to increasing dietary levels of CSM was quadratic and the diet containing 27.5% CSM provided the best growth. At the higher level of dietary iron (671 mg/kg), there was a linear decrease in weight gain with increasing level of CSM and fish fed diets containing 0% and 55.0% CSM had the highest and lowest WG, respectively. Pooled FI was similar

Table 3
Mean weight gain, dry matter feed intake, feed efficiency ratio (dry matter basis) and survival of channel catfish fed diets containing various levels of cottonseed meal and iron for 10 weeks

CSM (% of diet)	Fe (mg/kg diet)	Weight gain (g)	Dry matter feed intake (g/fish)	Feed efficiency ratio (dry matter basis)	Survival (%)
0	40	25.62	39.88	0.64	99.17
	336	27.96	42.16	0.66	99.17
	671	32.95	44.26	0.74	99.17
27.5	40	34.26	44.47	0.77	99.17
	336	33.72	44.07	0.76	100.00
	671	32.14	42.25	0.76	97.50
55	40	26.20	42.11	0.63	98.40
	336	24.44	36.01	0.68	99.17
	671	24.71	39.12	0.63	98.33
Pooled SEM		1.21	1.39	0.02	1.00
CSM effect (P level)		0.0001	0.0030	0.0001	ns
0		28.84[b]	42.10[a]	0.68[b]	99.17
27.5		33.37[a]	43.60[a]	0.76[a]	98.89
55		25.11[c]	39.80[b]	0.64[b]	98.63
Fe effect (P level)		ns	ns	ns	ns
	40	28.69	42.15	0.68	98.91
	336	28.70	41.88	0.70	99.44
	671	29.93	40.74	0.71	98.33
CSM × Fe (P level)		0.0073	0.0258	ns	ns
		LIN Fe, CSM = 0 QUAD CSM, Fe = 40 QUAD CSM, Fe = 336 LIN CSM, Fe = 671	LIN Fe, CSM = 0 QUAD Fe, CSM = 55.0 QUAD Fe, CSM = 336 LIN CSM, Fe = 671		

CSM = cottonseed meal.
Fe = iron (values include iron supplied by trace mineral mix).
ns = not significant.
LIN = linear response.
QUAD = quadratic response.

for diets containing 0% and 27.5% CSM but was significantly lower for diets containing 55.0% CSM. Dietary levels of supplemental iron had no effect on FI but the interaction between CSM and iron was significant. For diets without CSM, there was a linear increased in FI with increasing dietary level of iron. For diets containing 55.0% CSM, the effect of increasing dietary iron on FI was quadratic with fish fed diet supplemented with 336 mg iron/kg having the lowest FI. At a dietary level of iron of 336 mg/kg diet, FI of the 0% and 27.5% CSM diets was similar but the value was significantly lower for the 55.0% CSM diet. At 671 mg/kg of dietary iron, FI linearly decreased with increasing

dietary level of CSM. FER was significantly better for diets containing 27.5% CSM. No significant differences were observed in FER for diets containing 0% and 55% CSM. Dietary levels of supplemental iron and the interaction between CSM and iron had no effect on FER.

Mean values for total cell count (TCC), red blood cell count (RBC), hematocrit (Ht) and hemoglobin (Hb) were not significantly affected by dietary level of CSM (Table 4). TCC and RBC values, however, were significantly affected by dietary level of iron. The pooled TCC and RBC of fish fed the 336 mg/kg iron diets were significantly higher than

Table 4
Mean total cell count, red blood cell count, hematocrit and hemoglobin of channel catfish fed diets containing various levels of cottonseed meal and iron for 10 weeks

CSM (% of diet)	Supplemental Fe (mg/kg diet)	Total cell count (10^6/μl)	Red blood cell (10^6/μl)	Hematocrit (%)	Hemoglobin (g/dl)
0	40	1.84	1.55	29.56	5.08
	336	2.21	1.87	28.00	6.03
	671	2.74	2.20	31.16	8.78
27.5	40	2.48	2.01	31.26	10.40
	336	2.53	2.19	30.56	8.63
	671	2.08	1.73	28.46	6.22
55.0	40	1.92	1.59	28.70	8.41
	336	2.51	2.15	29.66	7.53
	671	2.18	1.84	28.50	6.32
Pooled SEM		3.08	0.10	1.50	0.89
CSM effect (P level)		ns	ns	ns	ns
0		2.26	1.87	29.58	6.63
27.5		2.36	1.98	30.10	8.41
5.0		2.20	1.86	28.95	7.42
Fe effect (P level)		0.0325	0.0027	ns	ns
	40	2.08b	1.72b	29.84	7.96
	336	2.42a	2.07a	29.41	7.40
	671	2.33ab	1.92a	29.38	7.10
CSM × Fe (P level)		0.0039	0.0024	ns	0.0050
		LIN Fe, CSM = 0 QUAD Fe, CSM = 55.0 QUAD CSM, Fe = 40 LIN CSM, Fe = 671	LIN Fe, CSM = 0 QUAD Fe, CSM = 27.5 QUAD Fe, CSM = 55.0 QUAD CSM, Fe = 40 LIN CSM, Fe = 671		LIN Fe, CSM = 0 LIN Fe, CSM = 27.5 QUAD CSM, Fe = 40

CSM = cottonseed meal.
Fe = iron.
ns = not significant.

those of groups fed diets with 40 mg iron/kg. No significant differences were observed for the pooled TCC and RBC of fish fed diets supplemented with 336 and 671 mg iron/kg. Ht and Hb, however, were not affected by dietary iron level. Interactions between dietary levels of CSM and iron were significant for TCC, RBC and Hb but had no effect on Ht. Among diets containing no CSM, there was a linear increase in TCC, RBC and Hb in response to increasing dietary level of iron. At a CSM level of 55.0%, the effect of increasing level of dietary iron on TCC and RBC was quadratic and fish fed the diet supplemented with 336 mg iron/kg had the highest TCC and RBC. For diets containing 27.5% CSM, the response of RBC to increasing dietary level of iron was also quadratic with fish fed the 336 mg/kg iron diet having the highest RBC. At the same level of dietary CSM (27.5%), Hb value decreased linearly with increasing dietary level of iron. For diets supplemented with 40 mg/kg of iron, there were quadratic responses in TCC, RBC and Hb with increasing dietary levels of CSM. Maximum values for these

Table 5
Mean macrophage migration, chemotaxis ratio and antibody titer of channel catfish fed diets containing different levels of cottonseed meal and iron for 10 weeks

CSM (% of diet)	Supplemental Fe (mg/kg diet)	Mean macrophage migration[a]		Macrophage chemotaxis ratio	Log $_{10}$ of antibody titer[b]
		Control (0 μg exoantigen)	Exoantigen (50 μg exoantigen)		
0	40	1.53	3.02	0.67	1.57
	336	1.68	4.06	0.71	1.98
	671	1.64	4.18	0.71	1.62
27.5	40	1.44	4.18	0.75	3.10
	336	1.86	4.04	0.68	3.00
	671	1.69	4.15	0.71	2.80
55.0	40	1.47	4.62	0.76	2.72
	336	1.93	4.67	0.70	2.73
	671	1.55	5.17	0.75	2.58
Pooled SEM		0.21	0.30	0.02	0.17
CSM effect (P level)		ns	0.0019	ns	0.0001
0		1.61	3.73b	0.69	1.72b
27.5		1.64	4.11b	0.71	2.68a
55.0		1.67	4.75a	0.73	2.97a
Fe effect (P level)		ns	ns	ns	ns
	40	1.48	3.92	0.72	2.46
	336	1.81	4.23	0.69	2.57
	671	1.63	4.44	0.72	2.33
CSM × Fe (P level)		ns	ns	ns	ns

CSM = cottonseed meal.
Fe = iron.
ns = not significant.
[a] $N = 9$ determinations/treatment.
[b] $N = 12$ determinations/treatment.

parameters were observed at the 27.5% CSM level. At the highest level of dietary iron (671 mg/kg), there was a linear decrease in TCC and RBC with increasing level of CSM.

Mean macrophage migration in the absence or presence of exoantigen, macrophage chemotaxis ratio and antibody titers are presented in Table 5. Dietary level of CSM had no effect on macrophage migration in the absence of exoantigen or macrophage chemotaxis ratio but significantly affected macrophage migration in the presence of exoantigen and antibody titers. Pooled mean macrophage migration in the presence of exoantigen of fish fed the 55.0% CSM diets was significantly higher than those of fish fed diets without or with 27.5% CSM. Antibody titers of fish fed diets without CSM was significantly lower than those of fish fed the 27.5% or 55.0% CSM diets. No significant differences were observed among the values for fish fed the 27.5% or 55.0% CSM diets. Dietary levels of iron, and the interactions between dietary levels of CSM and iron had no effect on macrophage migration with or without the presence of exoantigen, chemotaxis ratio and antibody titers.

Mean number of days at which the first mortality occurred after *E. ictaluri* challenge and cumulative mortality at 15 days post-immersion challenge with *E. ictaluri* are shown in Table 6. The number of days at which the first mortality occurred after *E.*

Table 6
Means days of first death and cumulative mortality of channel catfish fed diets containing various levels of cottonseed meal and iron at 15 days post-immersion challenge with *E. ictaluri*

CSM (% of diet)	Supplemental Fe (mg/kg diet)	Days to first mortality	Cumulative mortality (%)
0	40	8.0	48.3
	336	6.6	66.7
	671	6.6	48.3
27.5	40	7.6	33.3
	336	6.0	35.0
	671	6.3	36.7
55.0	40	8.0	18.3
	336	6.3	20.0
	671	7.6	26.7
Pooled SEM		0.81	9.68
CSM effect (*P* level)		ns	0.0023
0		7.1	54.4a
27.5		6.6	35.0b
55.0		7.3	21.6b
Fe effect (*P* level)		ns	ns
	40	7.8	33.3
	336	6.3	40.5
	671	6.8	37.2
CSM × Fe (*P* level)		ns	ns

CSM = cottonseed meal.
Fe = iron.
ns = not significant.

ictaluri challenge was not affected by dietary level of iron, CSM or the interactions between dietary level of iron and CSM. Cumulative mortality 15 days post-challenge was likewise not affected by supplemental level of iron or the interactions between dietary level of iron and CSM. However, cumulative mortality was significant affected by dietary level of CSM with fish fed diets without CSM having the highest mortality. No significant differences were observed for fish fed diets containing 27.5% or 55.0% CSM.

4. Discussion

Early studies have indicated that the amount of CSM that can be used in channel catfish feeds depends mainly on the level of free gossypol and available lysine content of the meal. Dorsa et al. (1982) found that 17.4% glanded CSM (0.49% free gossypol) can be included in channel catfish diets without adverse effects on growth. With low-gossypol CSM (0.022% free gossypol), a level of 25–30% can be used in channel catfish diets without detrimental effects (Robinson, 1991). He suggested that up to 30% CSM as a replacement of SBM can be used without adding lysine and that 100% of SBM can be replaced with CSM if supplemental lysine is used. In the present study, regardless of supplemental level of iron, fish fed diets containing 27.5% CSM (0.122% free gossypol) supplemented with lysine to a level equal to that of the SBM diets exhibited better WG and FER than those fed the control (0% CSM) and 55.0% CSM diets. Although a diet containing 27.5% CSM without supplemental lysine was not included, the improved performance of diets containing 27.5% CSM with added lysine obtained in our study but not in that of Robinson (1991) may be due to the differences in the free gossypol content of CSM. The CSM used in the present study contained about 5.5 times more free gossypol than that used in Robinson's studies. Free gossypol is known to bind lysine rendering it less bioavailable (Wilson et al., 1981).

Robinson and Rawles (1983) showed that supplementation of lysine to the diets containing 44.6% glandless cottonseed flour or 54.4% glandless cottonseed meal as total replacement of SBM did not improve growth and feed conversion. Glandless CSM which contain less than 0.01% free gossypol may contain sufficient lysine for channel catfish (Robinson and Li, 1995). However, when glanded CSM with 0.022% free gossypol was used to totally replace SBM, supplementation of lysine is needed to improve the nutritional value of channel catfish feed to a level comparable to that of the control (Robinson, 1991). In our study, even though lysine was added (total dietary lysine content of 6.2% of dietary protein), total replacement of SBM with CSM (0.122% free gossypol) resulted in reduced WG and FI. The higher concentration of free gossypol in CSM used in our study may account for differences between the results of this study and that of Robinson. Thus, the decreased WG and reduced FI of fish fed the 55.0% CSM diets containing 671 mg free gossypol/kg diet observed in our study may have been due to the high level of gossypol or some other unknown reasons. For example, Dorsa et al. (1982) reported that the growth of channel catfish was reduced when fed diets with 900 mg or greater free gossypol/kg diet. Fish strain and feed allowance may account for the differences between the results of these studies. In our study, fish were

fed to apparent satiation twice daily, whereas in the study of Dorsa et al. (1982), fish were fed 3% of body weight, divided into three equal feedings.

The response of fish, based on WG and FI, to various dietary levels of CSM is influenced by dietary iron concentrations. There was a linear increased in WG and FI with increasing level of iron for diets containing no CSM. This effect was not observed for diets containing 27.5% and 55.0% CSM as replacements of 50% and 100% SBM. The improved performance of SBM-based diets with increasing dietary iron levels can not be explained since the basal diet supplemented only with trace mineral mix (40 mg iron/kg) contained 213 mg iron/kg (Table 1) which is far greater than the reported 30 mg iron/kg diet required by channel catfish (Gatlin and Wilson, 1986; Lim et al., 1996b).

Few studies have been conducted to evaluate the effect of dietary CSM on fish hematological factors. Robinson and Li (1997) reported no differences in Ht of pond-raised channel catfish fed diets containing 16–32% crude protein with CSM levels ranging from 0% to 10%. In rainbow trout, Herman (1970) observed that Ht and Hb of fish fed a vitamin-free casein diet were not significantly different from those of fish fed diets containing 25% CSM from various sources (glandless meal, heated meal, commercial meal and raw meal) with dietary levels of free gossypol ranging from 0 to 290 mg/kg diet. However, Ht and Hb values were lower for fish fed the low-heat CSM diet (303 mg free gossypol/kg diet) or diet containing 1000 mg free gossypol/kg diet. Results of our study, however, showed that hematological factors (TCC, RBC, Ht and Hb) of juvenile channel catfish were not affected by dietary levels of CSM of up to 55.0% (671 mg free gossypol/kg diet), even though this level of CSM reduced growth performance.

The response of fish, based on TCC, RBC and Hb, to different dietary levels of CSM was influenced by supplemental levels of dietary iron. For diets containing no CSM (SBM-based diets), there was a linear increase in these parameters with increasing levels of iron supplementation. When SBM was totally replaced by CSM (55.0%), the diet supplemented with 336 mg/kg exhibited maximum TCC and RBC. For diets containing 27.5% CSM, maximum RBC was also obtained when 336 mg iron/kg was added. At the same level (27.5%) of CSM, there was a linear decrease in Hb value with increasing dietary level of iron. When 40 mg supplemental iron/kg was used, maximum TCC, RBC and Hb were obtained for a diet containing 27.5% CSM. At the highest level of supplemental iron (671 mg/kg), there was a linear decrease in TCC and RBC with increasing dietary levels of CSM. With pigs and broilers, addition of iron sulfate at a weight ratio of 1:1 of iron to free gossypol was effective in reducing the toxicity of free gossypol and improving their performances (Rojas and Scott, 1969; Wedegaertner, 1981; Jones, 1987). It was suggested that iron inactivates gossypol by forming a strong complex in the intestinal tract, thus preventing it from being absorbed (Wedegaertner, 1981). Thus, it was assumed that a higher level of iron supplementation is needed for diets containing CSM. However, this study showed otherwise, for maximum hematological values, higher level of supplemental iron was required for diets containing no CSM (SBM-based diets). Diets high in SBM may contain compounds or factors which reduce iron absorption or availability. Morck et al. (1982) reported that soybean products have a pronounced inhibitory effect on the absorption of nonheme dietary iron in men. Additional studies to determine the effect of iron supplementation to practical diets on the performance of

channel catfish are needed. Compounds or factors in practical ingredients affecting iron requirement and availability also need to be identified.

Mean macrophage migration in the presence of exoantigen was enhanced with increasing levels of dietary CSM as replacements for SBM. However, the effect became significant only when SBM was totally replaced by 55.0% CSM. Significant improvements in agglutinating antibody titers were also observed in fish fed diets containing CSM. Cotton seed meal may contain compounds which have immunostimulatory effects on both specific and non-specific immune response. Investigations to evaluate these hypotheses are warranted.

Channel catfish fed iron-deficient diets have been shown to have low macrophage migration in the presence of *E. ictaluri* exoantigen (Lim and Klesius, 1997; Sealey et al., 1997). However, engulfment of bacteria by macrophages as measured by chemiluminescent assay and antibody titers in response to formalin-killed *E. ictaluri* were not suppressed by iron deficiency (Sealey et al., 1997). Berger (1996) suggested that either a deficiency or excess of iron could compromise the immune system. In this study, supplementation of dietary iron to practical diets had no effect on macrophage migration or agglutinating antibody titers. Thus, iron present in practical diets, even not sufficient for maximum hematological factors, was probably sufficient to maintain normal function of the immune system. Sealey et al. (1997) reported that, depending on the bioavailability of iron in commercial feeds, actual levels may be at or above levels required for optimum immune response against *E. ictaluri*.

Sealey et al. (1997) showed that iron-deficiency increased susceptibility of channel catfish to *E. ictaluri* infection, but increasing dietary levels of supplemental iron to 180 mg/kg may lead to increased susceptibility. Lim and Klesius (1997) found that dietary iron did not protect against mortality of channel catfish from *E. ictaluri*. However, they observed that the onset of mortality of iron-deficient fish was earlier than those fed the iron-replete diet due to synergistic effect of iron deficiency and *E. ictaluri* infection. Data of our challenge study showed that iron supplementation at levels of 40–671 mg/kg to practical diets which contain approximately 204–283 mg iron/kg had no effect on the onset of mortality or cumulative mortality after *E. ictaluri* exposure.

Replacement of SBM with CSM significantly reduced cumulative mortality of fish 15 days post-challenge. Whether CSM contains some compounds, such as gossypol, that enhanced the resistance of fish to *E. ictaluri* infection is not known. It has been reported that substitution of corn gluten meal with CSM in diets fed to Pacific salmon produced similar growth, but the incidence of mortality due to kidney disease was much greater for fish fed the corn gluten meal diet as compared to those fed the CSM diet (Wood, 1968). Gossypol has been shown to immobilize and lyse African trypanosomes (*Trypanosoma brucei brucei*), in vitro, by interfering with glycolysis in the parasite (Eid et al., 1988). It has also been shown to be effective against two other parasites, *T. cruzi*, the cause of Chagas' disease and *Plasmodium falciparum*, the cause of malaria (cited by Eid et al., 1988). Polsky et al. (1989) also reported that gossypol inactivated human immunodeficiency virus in an in vitro system. It was observed in the present study that, after mortality due to *E. ictaluri* infection began to occur, fish fed the CSM diets (including those fed the highest CSM diets which had significantly reduced FI) continued to eat vigorously as compared to those fed diets containing no CSM (SBM diets). The increased survival and

the continued consumption of the CSM containing diets may be an indication of improved health status of fish fed these diets. However, further studies to evaluate the beneficial effect of gossypol and other compounds in CSM on fish immunity and disease resistance are needed.

Results of this study indicate that diets in which 50% of SBM was replaced by 27.5% CSM and lysine provided the best performance. Total replacement of SBM with 55.0% CSM (671 mg free gossypol/kg diet) reduced the nutritional value of the diet. For CSM-containing diets, supplementation of iron as ferrous sulfate at 1:1 weight ratio of iron to free gossypol had no effect on the nutritional value of the diet. However, supplementation of SBM-based diet with iron up to 671 mg/kg improved the nutritional value of the diet. Iron present in practical diets (203–283 mg/kg diet) appears to be sufficient to maintain normal immune function and protection against enteric septicemia of catfish caused by *E. ictaluri*. Gossypol or other compounds present in CSM may have a beneficial effect by improving the immune response and the resistance of juvenile channel catfish against *E. ictaluri* infection as evidence by increased macrophage chemotaxis, improved survival and continued consumption of diets containing CSM.

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